REGULATION OF CELL PROLIFERATION BY EPIDERMAL GROWTH FACTOR

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I. INTRODUCTION

In the last decade it became clear that the proliferation of cells in vivo and in vitro is controlled by several hormones and growth factors which are present in serum and in other tissue fluids. Since most of the growth factors are present in tissue fluids at very low concentrations, it is difficult to purify them in quantities which allow the characterization of their properties. Nevertheless, several growth factors have now been isolated and purified; the better characterized growth factors are nerve growth factor (NGF), epidermal growth factor (EGF), fibroblast growth factors (FGF), and platelet derived growth factor (PDGF).[1-4] Other growth factors which play an important role in the proliferation and differentiation of the hematopoietic system (e.g., colony stimulating factor (CSF) and macrophage-granulocyte inducer (MGI)[5,6] or of the immune system (e.g., interleukins, T-cell growth factors and others)[7,8] are not yet characterized to a similar extent.

Interest in growth factors was greatly stimulated by the finding that transformed cells generally have lower serum requirements than their normal counterparts. This led many investigators to the idea that study of the growth factors of normal and transformed cells in vitro might shed light on the control of normal and malignant growth in vivo. Since then several studies have shown that certain transformed cells produce growth factors which bind to membrane receptors and therefore reduce the binding of the exogenous growth factors to their membrane receptors. Todaro et al.[9] have shown that cells transformed by the RNA murine or feline sarcoma viruses rapidly lose their ability to bind EGF; whereas cells transformed by the DNA tumor virus, polyoma, and SV40, or infected with nontransforming RNA tumor viruses have normal levels of functional EGF receptors.[9] Subsequently it was shown that the virally transformed cells produce an "EGF-like" substance which competes with the authentic EGF for EGF receptors.[10] Therefore, this factor was called sarcoma growth factor (SGF). Todaro et al. postulated that the transformed cells which produce the "EGF-like" factor would be permanently stimulated to grow and will need less of exogenous growth factors for their proliferation. Similar growth factors termed "transforming growth factors" (TGF) were found in the conditioned medium of various cultured transformed cells,[11-14] in fetal calf serum[15] and in mouse embryos.[16] These TGFs have the following properties: they interact with EGF receptors and are strong mitogens which induce the overgrowth of cells in monolayer cultures. Moreover, they induce the morphologic transformation of normal cells and

anchorage independent growth: a property in cell culture that correlates best with tumorigenicity in vivo.[17] However, the TGFs do not cross-react with anti-EGF antibodies suggesting structural differences between EGF and the TGFs. It is not yet known whether all the effects of TGF are mediated via EGF receptor.

In this review we summarized studies which explore the molecular mechanism of the action of EGF. Our goal is to analyze various questions of mechanism concerning the action and regulation of EGF-receptor complexes leading to the repertoire of biological responses mediated by the growth factor. We would like to bring to the attention of the reader two recent reviews by Gospodarowicz[18] and Carpenter and Cohen,[19] which summarize important aspects of the biological effects, action, and regulation of EGF.

Mouse EGF is a single chain polypeptide of 53 amino acid residues (MW 6045). It is isolated from the submaxillary glands of adult male mice where it is found at levels of approximately 0.5% of the dry weight protein.[1,19] Human EGF (urogastrone) is isolated from human urine.[20,21] The amino acid sequence of human-EGF urogastrone is very similar to the sequence of mouse EGF, although it has a smaller molecular weight (5400 daltons). Both mouse and human EGF bind to the same receptor sites on mouse, human, rat, and chick cells.[1,18] The biological activity of mouse and human EGF is identical.

Various tissues respond to EGF. EGF stimulates the proliferation of the corneal epithelium and the epidermis. In skin, EGF induces the growth of epidermal and epithelial cells leading to an increase in the thickness of the epidermis accompanied by a decrease in fat. Other tissues which respond to EGF include the liver, lung, and kidney. EGF also stimulates the growth of organ cultures from mammary gland epithelial tissue. Another response of EGF is the inhibition of the secretion of gastric acid which is mediated by various types of stimuli.[20] EGF has also some tumor promoting activity, as it enhances the carcinogenicity of 3-methylcholanthene and potentiates the tumorigenicity of Kirsten Sarcoma virus.[22,23]

EGF stimulates the proliferation of various cultured cells from many different species.[1] In addition to its mitogenic response, EGF induces various early and delayed responses. Early responses include stimulation of ion and nutrient transport[1,19,24] enhancement of the phosphorylation of endogenous membrane proteins,[25] induction of specific changes in the organization of the cytoskeleton,[26] and changes in cell morphology.[27] Delayed responses include the activation of the enzyme ornithine decarboxylase[28] and enhancement of the biosynthesis of fibronectin[29] and keratin.[30] Like other growth factors, EGF induces a variety of cellular responses, the so called "pleiotropic response". The relationship between the early and delayed responses mediated by EGF is not known.

Maximal stimulation of DNA synthesis is achieved at partial occupancy of EGF receptors. Occupation of not more than 25% of the available receptors on the cell surface gives rise to maximal stimulation of DNA synthesis. The induction of DNA synthesis by EGF starts approximately 15 hr after the exposure to the growth factor and reaches maximal value after about 24 hr.[1,19,31] Several studies have shown that EGF must be continuously present in the medium for about 5 to 8 hr in order to initiate at least a partial enhancement of DNA synthesis. However, removal of EGF from the medium after 14 hr does not affect DNA synthesis measured at 24 hr after the addition of EGF. After 15 hr exposure to the hormone the cells become "committed" for DNA synthesis (for review see Reference 1).

II. A COMMON PATHWAY FOR THE REGULATION OF RECEPTORS FOR POLYPEPTIDE HORMONES

Cells which are exposed to increased concentrations of EGF or other hormones such as insulin or nerve growth factor (NGF) gradually lose a substantial fraction of their

receptors for the respective hormones.[1,3,19,32-34] This phenomenon called "down regulation" depends on hormone concentration, time, and temperature. When the cells are returned to medium free of hormone, the number of receptors returns to normal within 8 to 16 hr. Hormone induced receptor loss is usually accompanied by the appearance of degradative products of the cell associated hormone. Carpenter and Cohen[34] followed the fate of ^{125}I-EGF after its binding to cell surface receptors. They have shown that the binding of ^{125}I-EGF at 37° C is followed by rapid internalization, degradation in lysosomes, and release of ^{125}I-iodotyrosine from the cells. At 4° C the radiolabeled hormone remains associated with the cell surface. Moreover, the degradation of the internalized ^{125}I-EGF is blocked by chloroquine, ammonium ions, local anesthetics, and metabolic inhibitors.[34] The biological role of EGF internalization and degradation is not known. Aharonov et al. demonstrated[35] that the initial internalization of EGF and receptor down regulation are not sufficient for EGF mitogenesis. They suggested that the major role of the down regulation of EGF receptors is to adjust the cell's sensitivity to EGF.

The fate of EGF after binding to cell surface receptors was investigated in several laboratories by different techniques. Fluorescence microscopy and fluorescence photobleaching recovery (FPR) were used to trace the distribution and measure the mobility of fluorescent conjugate of EGF on living cells.[36-38] Electron microscopy was used to follow the fate of ferritin EGF[39] and colloidal gold avidin EGF[40] and electron microscope autoradiography was used to visualize the internalization of ^{125}I-EGF.[41]

The picture which emerges from these studies indicates that EGF becomes internalized by a process called receptor mediated endocytosis. EGF binds to diffusely distributed[36-39] laterally mobile[38] membrane receptors which rapidly cluster,[36-40] in a temperature dependent process, and become endocytosed and degraded by lysosomal enzymes.[41]

Receptor clustering and receptor mediated endocytosis are temperature-dependent processes which are completely inhibited at 4° C.[36-42] If receptor clustering and subsequent receptor mediated endocytosis occur at specific sites on the cell surface (i.e., coated pit areas), its inhibition at low temperature could be due to decreased lateral diffusion of the occupied EGF receptors. To test these possibilities Hillman and Schlessinger measured the lateral diffusion of rhodamine labeled EGF (R-EGF) bound to EGF receptors on human epidermoid carcinoma cells as a function of temperature. The value of D increased gradually from $D = 3 \times 10^{-10}$ cm^2/sec at 4° C up to $D = 8.5 \times 10^{-10}$ cm^2/sec at 37° C.[43] No phase transition was observed in this temperature range as previously reported for the lateral diffusion of various proteins incorporated in artificial lipid bilayers.[44] An activation energy of 6 kcal/mol was calculated for the temperature dependence of the lateral diffusion of EGF-receptor complexes;[43] a value similar to the activation energies measured for membrane proteins incorporated in artificial bilayers. Hillman and Schlessinger compared the temperature dependence of the lateral diffusion of EGF receptor complexes to the temperature dependence of the onset of patch formation and rate of internalization of ^{125}I-EGF into A-431 cells.[43] Figure 1 depicts the effect of temperature on the lateral diffusion of EGF-receptor complexes and on the rate of internalization of ^{125}I-EGF into these cells. Interestingly, at 15° C the internalization of EGF is very slow, while the lateral diffusion D is only half of the D value at 37° C.[43] From the calculation of the collision frequency of the occupied EGF receptors with coated regions using the measured values of D at 4° C and at 37° C, it was concluded that lateral diffusion is not the rate limiting step for either endocytosis or patching.[43] Moreover, since the internalization of radiolabeled EGF receptors precedes the formation of "visible patches" it was suggested that the bright patches observed by light microscopy are endocytic vesicles formed by the coalescence of several vesicles, each containing microclusters of EGF-receptor complexes.[42,43]

A new approach to study the dynamic properties of membrane receptors is to measure

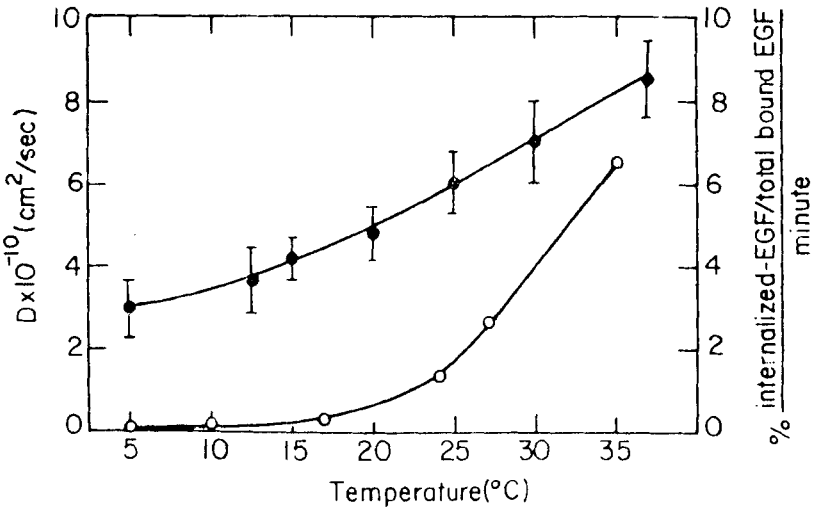

FIGURE 1. The effect of temperature on the lateral diffusion of EGF receptors (●) and on the initial rate of EGF internalization (o) into-431 cells.

their rotational diffusion by the method of time resolved phosphorescence emission and anisotropy.[45] Zidovetzki et al. used this method to measure the rotational diffusion of EGF-receptor complexes on human epidermoid carcinoma cells (A-431) under various conditions.[46] A biologically active phosphorescent conjugate of EGF, erythroscei EGF, was applied to the cells. The EGF-receptor complexes are mobile with rotational correlation times in the range of 25 to 50μsec when labeled at 4°C. Prolonged incubations or exposure to higher temperature resulted in longer times up to 350μsec, indicative of progressive formation of microclusters, estimated to contain 10 to 50 receptors. Upon internalization and the formation of visible patches of EGF-receptor complexes, the rotational correlation times were shorter, indicating a decrease in size of the dynamic unit. The sign of the rotational relaxation also varied with the internalization and processing of the hormone.

While the lateral diffusion coefficient of the EGF-receptor complex increased upon heating from 4°C to 37°C, the rotational diffusion decreased in the same range of temperatures. According to the theory of Saffman and Delbruck,[47] the rotational diffusion coefficient is a sensitive measure of the radius of the rotating molecule while the lateral diffusion coefficient is relatively insensitive to the radius of the diffusing entity. Moreover, both the lateral and the rotational diffusion should increase with temperature as a function mainly of $T/\eta(T)$ where T is the absolute temperature and $\eta(T)$ is the viscosity of the lipid matrix. However, if microclusters are formed at 37°C, the retardation contributed by the clustered receptors could outweigh the contribution due to increased kinetic energy and decreased membrane viscosity. Hence, the microclusters would rotate slower at 37°C than individual receptors at 4°C. In contrast, since the lateral diffusion is not sensitive to the size of the diffusing molecules, both individual EGF receptors and microclusters would diffuse at similar rates which increase with temperature. This is what was observed.[43]

On the basis of reports from various laboratories which used different techniques it is possible to deduce the following picture concerning the fate of EGF after its binding to cell surface receptors.[36-43,46] Figure 2 depicts steps in the internalization of EGF.

1. EGF binds to diffusely distributed membrane receptors which translate and rotate rapidly in the plane of the membrane.

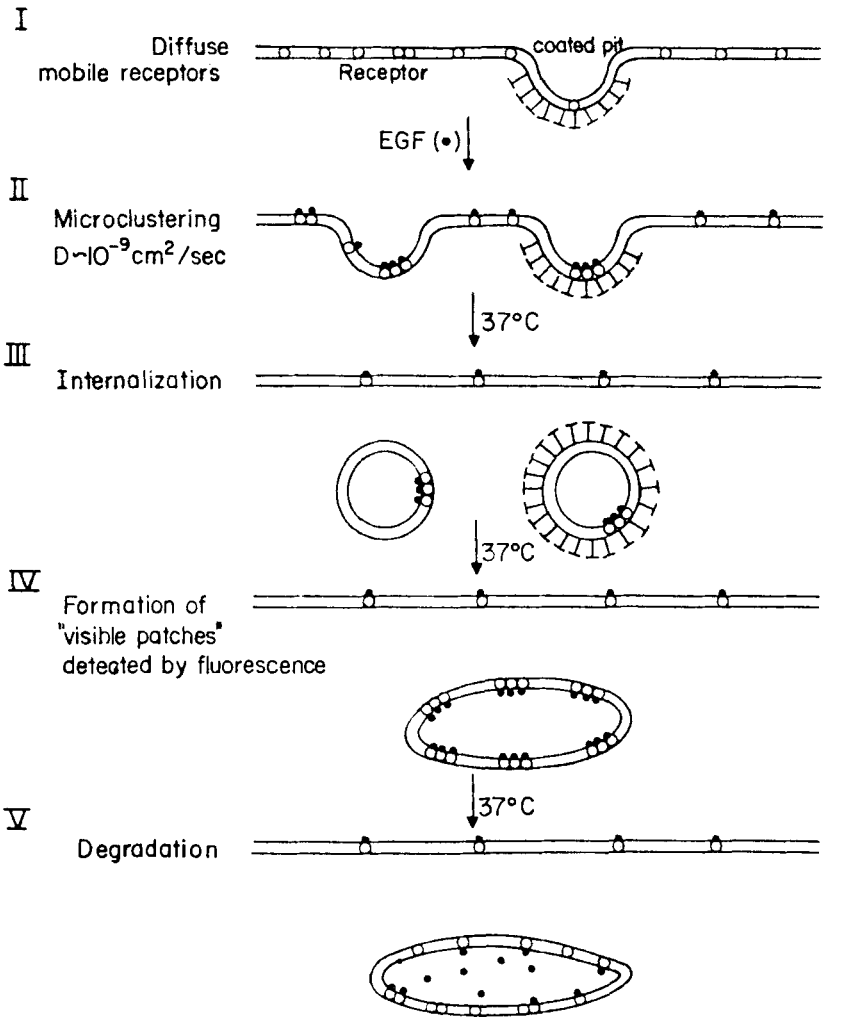

FIGURE 2. Steps in the internalization of EGF. (I) EGF binds to diffusely distributed receptors which translate and rotate rapidly in the plane of the membrane. (II) At 37°C, EGF induces the microclustering of receptors in non-coated and coated areas on the cell surface. (III) Coated and non-coated vesicles are formed at 37°C containing microclusters of EGF-receptor complexes. (IV) "Visible patches" of EGF-receptor complexes detected by fluroescence microscopy are formed intracellulary. (V) EGF is degraded by lysosomal enzymes.

2. Increasing the temperature leads to the formation of microclusters which rotate slower and translate faster at 37°C compared to their respective rates of rotation and translation at 4°C. The clustering seems to occur on coated pits and on non-coated areas.[39,41]
3. The clustered EGF-receptor complexes pinch off, by an energy dependent process, and form coated (and non-coated) vesicles.
4. The endocytic vesicles are processed inside the cell and EGF and presumably also the receptor molecule are degraded by lysosomal enzymes.

The biological role of each of these steps is not clear. It is possible however, to raise at least three different mechanisms for the transduction of its biological activity.

1. EGF acts at the level of the cell surface to generate directly or indirectly via a "second messenger" (e.g., phosphorylation of intracellular proteins, concentration of ions and metabolites), a signal that triggers both rapid and delayed responses. Alternatively, one or more of the rapid responses may produce the signal for the long-term effects. In this model internalization does not participate in the signalling process, it is rather related to the desensitization of the cell to the growth factor.
2. EGF generates a signal at the level of the plasma membrane for the rapid responses, and the internalized hormone or its fragments produce an independent signal by interaction with putative intracellular sites for the long-term effects.
3. EGF binding to cell surface receptors creates the appropriate perturbation in the receptor molecule which by itself is the active species.

Obviously it is possible to suggest additional mechanisms. Nevertheless, these three alternatives set the stage for systematic investigation of the mechanism of EGF action. In the next section we discuss various questions concerning the mechanism of EGF response which shed light on the three alternative mechanism proposed below.

III. A POSSIBLE ROLE FOR THE MICROAGGREGATION OF EGF RECEPTORS

EGF has a single methionyl at position 21. Cleavage with cyanogen bromide results in the formation of two polypeptide chains connected by the three disulphide bonds of the molecule.[48,49] CNBr-cleaved EGF (CNBr-EGF) retains approximately 10% of the binding activity of EGF but is virtually devoid of mitogenic activity in vitro[48,50,51] and in vivo.[49] It is not known yet whether the non-mitogenic property of CNBr-EGF is due to the cleavage at the methionyl residue per se or due to further changes induced in the EGF molecule during the reaction with cyanogen bromide.

In contrast to EGF, CNBr-EGF does not induce the clustering of the EGF-receptor complexes. However, the addition of bivalent anti-EGF antibodies restores both patch formation and the mitogenic activity of this analogue.[48] The mitogenic activity is not restored by adding monovalent Fab′ fragments of the antibodies. The concentration of bivalent antibodies required for full restoration of DNA synthesis is far below the concentration at which visible patches of R-EGF can be seen. Thus, it was concluded that microaggregation of perhaps only a few receptors rather than the formation of visible patches may be the relevant mechanism for the induction of the mitogenic activity of EGF.[33,48,52]

Two additional experiments demonstrate the significance of receptor clustering. First, concentrations of native EGF far below those that normally show activity can elicit dramatic responses when bivalent anti-EGF antibodies are added. Monovalent antibody fragments cannot enhance DNA synthesis. Second, cells that are relatively refractory to EGF, despite unimpaired binding, can be markedly sensitized and respond to the hormone after cross-linking with anti-EGF antibodies.[48] Thus it was proposed that receptor aggregation potentiates the biological response of EGF.[33,48,52] It was previously argued that ligand induced receptor clustering could cause an efficient amplification of biological signals across the plasma membrane of various ligand-receptor systems.[52]

An alternative interpretation of the effect of anti-EGF antibodies on the response of EGF is that the bivalent anti-EGF antibodies enhance the apparent affinity of EGF towards its receptors rather than enhance receptor clustering. The binding constant (K) of EGF to EGF receptor equals k_{on}/k_{off} where:

$$\text{EGF} + \text{Receptor} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{EGF} - \text{Receptor}$$

Anti-EGF antibodies are able to increase the binding constant K by either increasing k_{on} or by decreasing k_{off} (or both). Since the cells are exposed to EGF in the presence of the antibodies it is impossible to rule out a direct effect on the k_{on}. Moreover, the effect of anti-EGF antibodies on the k_{off} was not studied in detail. Therefore it is required to study the effect of anti-EGF antibodies on the binding parameters of EGF in order to understand the antibody-induced potentiation of the biological response to EGF.

IV. EGF RECEPTOR AND EGF INDUCED-PROTEIN PHOSPHORYLATION

The membrane receptor of EGF plays a key role in the transduction of the signals mediated by EGF leading to cell proliferation. The EGF receptor interacts with transforming growth factors (TGF) leading to anchorage-independent growth.[9-16] Tumor promoters such as phorbol esters[53] induce a decrease in the affinity of EGF towards its receptors. Recent studies indicate that EGF receptor could also interact with receptors for vasopressin on 3T3 cells.[54] Schreiber et al. have recently reported on the generation of a monoclonal antibody against EGF receptor.[55] This antibody blocks the binding of radiolabeled EGF to EGF receptors on 3T3 cells, human fibroblasts, and A-431 cells.[55] Interestingly it also induces "EGF-like" activity: the phosphorylation of membrane proteins, activation of ornithine decarboxylase, and the stimulation of DNA synthesis and cell proliferation.[55,56] These results support the notion that EGF receptor rather than EGF itself is the active moiety and that the role of the hormone is to perturb the receptor which then stimulates the pleiotropic responses mediated by EGF.

EGF receptor is a membrane glycoprotein containing sialic acid residues. EGF binds to the EGF receptor with an apparent dissociation constant of 2×10^{-10} *M*. Most cells which are responsive to EGF bear 40,000 to 100,000 EGF receptors per cell. A cell line with an unusually high number of EGF receptors which is commonly used for the investigation of EGF receptors is the human epidermoid carcinoma A-431 which bears approximately 2×10^6 receptors per cell.[57]

Various approaches have been used to characterize and isolate the receptor for EGF. Cross-linking reagents have been used to produce covalent EGF-receptor complexes of approximately 190,000 daltons from 3T3 fibroblasts[58] and 100,000 daltons from liver membranes.[59] Linsley et al. have shown that a fraction of ^{125}I-EGF forms a direct-linkage complex with EGF receptors.[60] This "covalent association" of EGF receptor appears to be due to an oxidative effect on EGF by chloramine T.[61] Similar direct linkages were reported for ^{125}I-insulin and ^{125}I-thrombin with their respective receptors.[62,63] Anti-EGF antibodies were used for the immunoprecipitation of ^{125}I-EGF directly linked receptor complex. The immunoprecipitated receptor appeared as a 160,000 to 140,000 daltons proteins.[64] EGF-affinity chromatography of EGF receptor from membranes prepared from A-431 cells indicated that EGF receptors is a protein of 170,000 daltons.[65]

Although limited progress has been made in purifying the EGF receptor by solubilization of membranes, much more progress was made in an attempt to obtain a cell-free system which is responsive to EGF. Carpenter and Cohen discovered that EGF induces a rapid, cyclic nucleotide-independent and tyrosine-specific phosphorylation of a number of membrane proteins when added to purified plasma membranes from human epidermoid carcinoma cells (A-431). Several proteins of molecular weights corresponding to 170,000, 150,000, 80,000, and 22,000 are phosphorylated.[66-69] The major phosphorylated proteins with molecular weight of 170,000 to 150,000 daltons were identified as EGF receptor.[66-69] Both ATP and GTP can act as the phosphate donor of the phosphorylation reaction and either Mn^{+2} or Mg^{+2} is required for optimal activity of the protein kinase.[68] The kinase activity is copurified with EGF-binding activity by EGF affinity-chromatography.[65] This finding together with the fact that the phosphorylation

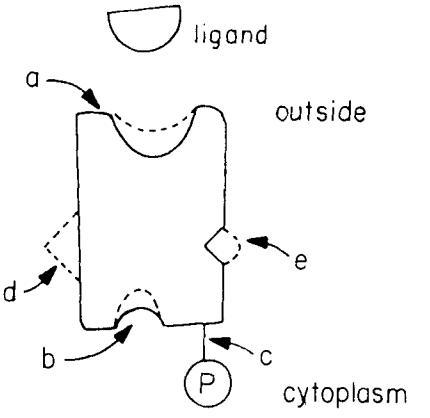

FIGURE 3. A hypothetical model describing EGF receptor as an "allosteric receptor" composed of various functional sites. (Site a) combining site for the various polypeptide ligands EGF, CNBr-EGF, TGF, and monoclonal antibodies against EGF receptors. All these ligands serve as "allosteric regulators" of EGF receptors. (Site b) Tyrosine specific, cAMP independent, protein kinase. (Site c) Phosphorylation site(s) at tyrosine residue(s). (Site d) Aggregation site which facilitates receptor-receptor or receptor-coated pit interactions. (Site e) Modulation sites for interactions with other receptors known to modulate the binding and activity of EGF (i.e., TPA, vasopressin). It is postulated that the binding of the "allosteric regulators" to the combining site leads to a conformational change in the "allosteric receptor" which leads to the expression of the various functional sites. Continuous lines indicate the conformation of the sites in the absence of EGF and dotted lines represent the conformation of the sites in the presence of EGF.

is very rapid, even at 0° C, suggests a close proximity between the receptor and the kinase. In fact, Cohen et al.[70] provide strong evidence that the protein kinase is an integral part of EGF receptor by photoaffinity label of an ATP binding on the receptor molecule (see model in Figure 3) The kinase activity which is associated with EGF receptor is very similar to the kinase activity of several RNA tumor viruses. Interestingly, antibodies against the kinase of Rous sarcoma virus protein ($pp60^{src}$) are specifically phosphorylated when interacting with affinity-purified EGF-receptor kinase preparation. However, the anti-$pp60^{src}$ antibodies do not immunoprecipitate the EGF-receptor kinase.[71,72] These results suggest that the EGF-receptor kinase is related but probably not identical with $pp60^{arc}$.

Treatment of A-431 cells with EGF was shown to enhance the phosphorylation of several cellular proteins.[73-74] One of the phosphorylated proteins is the 34,000 dalton proteins which is the one of the main substrates for the protein kinase associated with the $pp60^{src}$.[73-74] Hence at least one of the proteins which are phosphorylated by the kinase associated with an oncogenic virus is also phosphorylated by the EGF-sensitive system.

The striking resemblance between the kinase activities associated with several oncogenic RNA viruses and with EGF receptors suggests that tyrosine-specific, cAMP-independent kinase, play an important role in the regulation of cellular growth (see Table 1). However, the result of Schreiber et al. indicate that the non-mitogenic CNBr-

Table 1
COMPARISON OF THE EFFECTS OF EGF AND TRANSFORMATION WITH SARCOMA VIRUS ON CERTAIN CELL PROPERTIES

Effects	EGF	Sarcoma virus	Ref.
Morphological changes	+	+	27
Reorganization of cytoskeletal elements			
Microfilament	+	+	26
Microtubules	−	−	26
Intermediate filaments	−	−	26
Activity associated with protein kinase			
Tyrosine specific	+	+	69
cAMP independent	+	+	65—68
Phosphorylation of 34,000 dalton protein	+	+	73, 74
Phosphorylation of antibodies against pp 60^{src}	+	+	71, 72

EGF is as potent as native EGF in enhancing the phosphorylation of endogenous membrane proteins prepared from A-431 cells.[50] This result suggests the following two alternative roles for the EGF-induced phosphorylation (1) it is not relevant for the triggering of DNA synthesis and (2) more likely, it is a necessary but not sufficient signal for the induction of DNA synthesis by EGF.[50]

On the basis of various studies described in the literature it is possible to draw an hypothetical model of EGF receptor. It is assumed that the 170,000 to 150,000 dalton receptor molecule spans the plasma membrane and that the kinase activity comprises an integral portion of EGF receptor, facing the cytoplasmic portion of the receptor molecule. Several laboratories provide data which is consistent with this notion,[65,70,73] nevertheless, they do not provide a definite proof to this assumption.

According to the model (see Figure 3) the binding of EGF or the other ligands which bind to the combining site (site a) or to an area close to it (i.e., TGF, CNBr-EGF, and monoclonal antibodies against EGF receptor) induce a conformational change in the receptor molecule. The conformational change in the receptor molecule enhances the activity of the protein kinase (site b). One of the proteins phosphorylated by the kinase is EGF receptor itself (site(s) c).

The conformational change in the EGF-receptor complex could lead to the appearance of an additional functional site, an *aggregation site* (site d). This site facilitates receptor-receptor or receptor-coated pits interactions.

As it is known that EGF receptor is capable of interacting with other receptors or membrane proteins such as vasopressin receptor,[54] TPA receptor,[76] and the major histocompatability complex.[77] It is possible that EGF receptor contains also a *modulation site* (site e) or modulation sites. Namely, sites which allow lateral traffic with other receptors which are known to modulate the binding and response to EGF.

In general terms we envision EGF receptor as an "allosteric" protein which is embedded in the plasma membrane, therefore we call such receptors "allosteric receptors". In such allosteric receptors the combining site for the ligands (allosteric regulators) is at the external portion of the receptor, while the functional sites are either separated by the plasma membrane as part of the cytoplasmic portion of the receptor molecule or within the lipid matrix.

Table 2
BIOCHEMICAL EFFECTS OF THE MONOCLONAL ANTIBODIES AGAINST EGF RECEPTOR

	Concentration of 2G2-IgM	Concentration of EGF
50% inhibition of binding of EGF (2nM)	20nM	2nM
Enhancement of phosphorylation of membrane proteins	50nM	20nM
Induction of changes in cell cell morphology	>50nM	>10nM
Activation of the enzyme ornithine decarboxylase	10nM	2nM
Maximal induction of DNA synthesis	10nM	2nM
Stimulation of cell proliferation	20nM	2nM

Thus, EGF and other ligands which bind to the combining site act as *allosteric regulators* inducing a conformational change in the *allosteric receptor* which lead to the expression and activation of new *functional sites.*

V. MONOCLONAL ANTIBODIES AGAINST EGF RECEPTOR INDUCE "EGF-LIKE" ACTIVITY IN RESPONSIVE CELLS

A powerful tool for the isolation and investigation of membrane receptors is to use specific antibodies directed against the membrane receptors. Haigler and Carpenter prepared antibodies directed against the membrane or human epidermoid carcinoma cells A-431.[78] They reported that these antibodies inhibit the binding of EGF to cells and membrane bearing EGF receptors. Hence, these antibodies inhibit EGF binding, and bioactivity and do not possess any intrinsic biological activity.

Schreiber et al. reported on the generation of monoclonal antibodies against EGF receptors.[55] They raised antibodies against A-431 cells and selected monoclonal antibodies against EGF receptors. One fast growing clone that secreted IgM antibodies (denoted 2G2) capable of inhibiting the binding of EGF to membrane receptors, was grown in culture in large quantities and in the form of ascites in mice.

The properties of this antibody are as follows.

1. 2G2 antibody binds to cells bearing EGF receptors in proportion to the amount of EGF receptors on these cells. 2G2 antibody inhibits specifically the binding of radiolabeled EGF to human, rat, and mouse cells and to membranes prepared from these cells. It does not bind to cells which do not bear EGF receptors.
2. 2G2 antibody induces various early and delayed responses which are mediated by EGF. This antibody induces the enhancement of phosphorilation of endogenous membrane proteins, changes in the morphology of A-431 cells, activation of the enzyme ornithine decarboxylase, stimulation of DNA synthesis in responsive cells, enhancement of the proliferation of cells bearing EGF receptors. Thus 2G2 antibody acts as a competitive agonist of EGF inducing both the early and the delayed effects mediated by the growth factor (see summary in Table 2).

In contrast to the potency of 2G2-IgM in inhibiting the binding of radiolabeled EGF to its membrane receptors, an excess of EGF could not displace the bound 2G2 from the membrane receptor.[55,59] However, monovalent Fab' fragments of 2G2 inhibit the binding

Table 3
PROPERTIES OF MONOCLONAL ANTIBODY AGAINST EGF-RECEPTORS AND ITS FAB' FRAGMENT

	Concentration of IgM-2G2	Concentration of Fab'-2G2
50% inhibition of the binding of ^{125}I-EGF (2nM)	10 nM	1 μM
Inhibition of the binding of IgM (10 nM) or Fab' (1μM) by EGF	no inhibition	5 nM
Maximal induction of DNA synthesis	10 nM	no stimulation

of ^{125}I-EGF and EGF blocks the binding of radiolabeled Fab'-2G2[56] (see Table 2). Schreiber et al. have shown that the dissociation constant of the decavalent IgM from the cell surface is very low. This could be due to the rapid cross-linking of mobile EGF receptors followed by the endocytosis of the EGF-receptor complex. Indeed the addition of rhodamine labeled 2G2-IgM to either A-431 cells or human foreskin fibroblasts is followed by rapid membrane clustering and endocytosis of the fluorescent antibody-receptor complex.[56] Upon incubation of 4°C, rhodamine-labeled 2G2-IgM appears in small bright spots and also diffusely distributed all over the cell surface. When the labeled cells were warmed to 37°C the fluorescent 2G2 is rapidly endocytosed into bright fluorescent patches. Hence, like EGF, 2G2-IgM antibodies cluster on the cell surface and become rapidly internalized into cells bearing EGF receptors.[56] However, the monovalent Fab fragments do not induce the clustering of EGF receptors.[56]

The "EGF-like" activity induced by the anti-EGF receptor antibody indicates an important feature of the EGF receptor complex system. It strongly supports the notion that the biological information of the EGF-receptor complex system resides in the receptor molecule and that the role of the ligand (i.e., EGF), is to perturb the *allosteric receptor* (see Figure 3) in a way which will lead to the pleiotropic responses to EGF.

It is noteworthy that heterogenous bivalent IgG antibodies which block the binding of radiolabeled EGF to membrane receptors are devoid of "EGF-like" activity.[78] This could be the consequence of several reasons. First, the decavalent IgM is likely to be a better cross-linking agent of the mobile EGF receptors[55,56] compared to the bivalent IgG antibodies. Second, the very low dissociation constant of the bound 2G2 monoclonal antibodies is also likely to be slower compared to the dissociation constant of the bivalent antibodies. Finally, it is possible that the monoclonal antibodies bind to the binding domain of EGF on the membrane receptors, hence providing the necessary perturbation in the receptor molecule which mimicks the perturbation mediated by EGF.

The first two explanations could also account for the non-mitogenic activity of the monovalent fragments of 2G2-IgM[56] (see Table 3). Namely, it cannot induce DNA-synthesis because of the following reasons:

1. The Fab fragment fails to cross-link EGF receptors or alternatively.
2. The affinity of this reagent towards EGF receptor is too low to provide the necessary perturbation in the receptor which is required for the stimulation of DNA synthesis.

The addition of antibodies against Fab' fragments to human foreskin fibroblasts which were initially labeled with the non-mitogenic Fab-2G2 leads to full restoration of

DNA synthesis. This experiment seems to suggest that receptor clustering is required for the restoration of DNA synthesis. However, it is also possible that the anti-Fab antibody enhances the affinity of the Fab' fragment towards EGF receptors.[56]

VI. POSSIBLE RELATIONSHIP BETWEEN EGF INTERNALIZATION AND ITS MITOGENIC EFFECT

Several studies support the idea that intracellular degradation of EGF or EGF receptors is necessary for the mitogenic response to EGF. Fox and Das have shown that at low concentration of EGF added to 3T3 fibroblasts the rate of receptor internalization and degradation correlates well with its mitogenic activity.[79] They have suggested that the proteolytically cleaved EGF receptor may be involved in the activation of DNA snythesis.[79] It is not clear yet whether the proteolytic fragments of EGF receptor are related to the EGF-induced protein activator of DNA synthesis which was isolated from cytoplasmic extracts of 3T3 cells treated with EGF.[80]

One approach to investigate the biological role of EGF internalization and degradation is to use drugs which inhibit specific stages in the processing of EGF-receptor complexes and then to examine their effect on the biological response mediated by EGF.

Various amines block the degradation of the endocytosed EGF without affecting the binding of the hormone to its surface receptors.[34] Several reports presented data indicating that primary amines inhibit the formation of visible patches on the cell surface of fluorescently labeled EGF, α_2-macroglobulin and 3,3',5-triiodo-L-thyronine and also their subsequent internalization into the cells.[81,82] Hence these drugs seem to provide a tool for investigating the biological role of hormone clustering and internalization. However, amines at doses which inhibit receptor clustering do not inhibit, and even potentiate EGF stimulated DNA synthesis by 3T3 cells.[83] This suggests that receptor clustering and internalization are related to the removal of the hormone from the cell surface rather than to the mechanism of stimulation.[83] Recently Haigler et al. reported that amines block the endocytosis of α_2-macroglobulin but do not block the endocytosis of EGF into 3T3 cells.[84] Previous studies have shown that amines did not inhibit the endocytosis EGF into human epidermoid carcinoma cells (A-431) and fibroblasts,[38] and that the main action of amines is inhibition of hormone degradation.[41,42,85,86]

Since various primary amines and inhibitors of the enzyme transglutaminase block the formation of visible patches of rhodamine α_2-macroglobulin and rhodamine EGF it was postulated that this enzyme cross-links covalently the receptors and putative proteins in the coated pits by forming ϵ-(γ-glutaminyl) lysine cross-bridges.[87] The conflicting reports concerning the effects of amines on EGF processing and activity led several laboratories to perform a comprehensive investigation of the effects of amines on the fate of EGF after the binding to cultured cells.[41,82,86,88] Current studies from several laboratories provide the following view on the effect of amines on EGF processing and action. Amines do not affect the rate and the extent of internalization of EGF into 3T3 cells. Studies with fluorescent conjugates of EGF show that amines slow the formation of visible patches of EGF on 3T3 cells.[42] Fluorescence photobleaching recovery (FPR) and video intensification microscopy cannot detect the formation of microclusters of EGF on the cell surface. However, Haigler et al. have shown by electron microscopy that ferritin EGF forms microclusters composed of 2 to 6 molecules at 37° C and that amines did not block this process.[39] Such microclusters are presumably mobile and therefore capable of coalescing into patches on the cell surface. However, several studies suggest that microclusters of EGF-receptor complexes are internalized and that the "visible patches" are in fact endocytic vesicles.[37,42,43] Therefore the effect of amines could be interpreted as

the inhibition of fusion between vesicles containing fluorescent hormone. It was reported that amines prevent the fusion between lysosomes and multivesicular bodies and between the membranes of semliki Forest Virus and the lysosome.[88] Thus, a more general effect of amines could be the inhibition of fusion between intracellular membranes.

Johnson et al. reported that inhibitors of lysosomal enzymes enhance the nuclear accumulation of EGF[89] and Fridkin et al. indicated that the inhibition of EGF degradation leads to enhanced DNA synthesis.[90] However, other investigators reported that the degradation of EGF is not related to the mitogenic effect of EGF.[91]

It is possible that the conflicting reports concerning the effects of various drugs on EGF processing and action are due to adverse effects of these drugs on various cellular functions. Thus, the role of endocytosis and intracellular degradation of EGF and of EGF receptor in the mitogenic response remains unresolved.

The biological activity of the anti-EGF receptor antibodies implies that endocytosis and degradation of EGF is not important in the biological response.[55,56] However, it is not known whether the internalization and degradation of EGF receptor is required for the mitogenic response.

VII. OTHER MOLECULES WHICH INTERACT WITH EGF RECEPTORS OR EFFECT ITS CELLULAR RESPONSE

EGF receptor serves as the binding site not only for EGF but also for other polypeptide growth factors. The most interesting polypeptides which bind to EGF receptors and modulate its response are the transforming growth factors (TGF). Todaro et al. have shown that cells transformed by murine sarcoma viruses have reduced or absent cell surface receptors for EGF, as compared to the untransformed counterparts or to cells transformed by other viruses.[9] It was shown that sarcoma virus transformed cells release into the cell culture medium a family of polypeptide growth factors which bind to EGF receptors and induce cell proliferation. The conditioned medium contains SGFs with molecular weights of approximately 7,000, 12,000, and 25,000 daltons. In addition to the mitogenic properties of EGF the SGF induce normal fibroblasts to grow in soft agar and to express phenotypic properties of transformed cells. The expression of the transformed phenotype mediated by SGF requires a continuous presence of this factor.[11]

Recently TGFs were shown to be produced by different human tumor lines[11-13] and by chemically transformed tumor cells.[11,13] Moreover, similar TGFs were detected in mouse embryos[15] and in fetal calf serum.[16] The TGFs from these various sources were isolated by an acid/ethanol extraction and they have the following properties. Their molecular weights are in the range of 10,000 to 24,000 daltons and they are heat stable polypeptides with disulfide bonds. All of them block the binding of ^{125}I-EGF to EGF receptors, induce DNA synthesis and morphological transformation as well as colony formation of normal cells in soft agar. The question remains still open whether all the biological effects of the TGFs are mediated via EGF receptor. Interestingly, antibodies against EGF *do not* cross-react with either TGF or SGF[9-16] suggesting that EGF is antigenically distinct from the various TGFs. The presence of these growth factors in mouse embryos and in fetal calf serum (but not in calf serum) suggest that both fetal development and neoplastic transformation may be affected by similar growth factors.[11] In fact, Sporn and Todaro suggested that malignant transformation by certain carcinogens or tumor viruses may result from inappropriate expression of growth factors that were required in normal early embryogenesis.[11] It is therefore of great interest to examine the possibility that TGFs and EGF belong to a family of related polypeptide growth factors.

The finding that 3T3 variants which do not possess EGF receptors can still be transformed by murine sarcoma virus[92] argues in favor of the notion that SGF (and other

TGFs) do not mediate all the effects of sarcoma virus transformation.[9-11,14] Hence it is possible that TGFs do not represent a major transforming activity mediated by sarcoma virus or that other unknown TGFs utilize receptors for other growth factors. Transformed cells also produce factors which enhance DNA synthesis and increase the binding of radiolabeled EGF to its membrane receptors.[93]

Moreover, Roberts et al. reported on a EGF-dependent TGF, namely a transforming growth factor isolated from tumor cells which requires EGF to induce colony formation of indicator cell in soft agar.[94] Cherington and Pardee have shown that transformed chinese hamster fibroblast (CHEF) lose their EGF requirement for growth.[95,96] Moreover, the transformed CHEF cells have fewer EGF receptors than their normal counterparts but they do not seem to produce a "TGF-like" material that could account for receptor loss. Therefore Cherington and Pardee propose that this phenotype is mediated by an intracellular process which leads to the reduction of the number of EGF receptors and not via the medium as with TGFs which block the binding of exogenous ^{125}I-EGF.[95]

It is noteworthy that EGF itself is capable of inducing certain aspects of the transformed phenotype.[25] EGF also enhances the incidence of transformation of granulosa cells by Kirsten sarcoma virus[23] and the chemical induction of skin tumors in mice.[22] EGF appears to act as a tumor promoter in retroviral and chemical induced transformation.

The tumor promoting phorbol ester 12-0-tetradecanyl phorbol-13-acetate (TPA) inhibits the binding of EGF to cellular receptors on various cell types. The inhibition of binding correlates well with the biological activity of various tumor promoters on mouse skin and in cell culture.[97-100]

The inhibition of EGF binding by TPA is a highly temperature dependent process, it occurs at 37°C but not at 4°C. Moreover, TPA binds to specific membrane receptors[100] which are distinct from EGF receptors. This suggests that TPA initially binds to receptors which are separate from EGF receptors and that the occupied TPA receptors interact with EGF receptors, thereby affecting EGF binding and activity in a temperature sensitive process (see model in Figure 3).

Similar inhibitions of the binding of EGF to its membrane receptors were observed when fibroblasts were treated with either vasopressin[54] or with saccharine.[101] It seems however that EGF receptor is capable of interacting with various membrane molecules which in turn can modulate the binding and response to EGF (Figure 3).

VIII. SUMMARY AND FUTURE PROSPECTS

In the last few years several discoveries provided important insights into the mechanism of action of EGF.

The pioneering discovery of Carpenter and Cohen that EGF activates a tyrosine-specific, cAMP-dependent, protein kinase provides the first cell free system responsive to EGF.[66] Moreover, the similarity between the EGF-sensitive kinase and the kinases associated with certain oncogenic viruses led to the notion that tyrosine-specific phosphorylation could act as a "second messenger" for the stimulation of cellular growth. It is therefore of great interest to identify the cellular proteins which are phosphorylated by the EGF-sensitive system and to examine the possibility that the phosphorylation of these proteins provides internal stimuli for the induction of the pleiotropic response of EGF.

The application of modern biophysical tools such as the methods of fluorescence photobleaching recovery, image intensification microscopy, measurements of rotational diffusion by the analysis of the decay of phosphorescent anisotropy, together with

detailed ultrastructural analysis of the fate of ferritin and radiolabeled EGF by electron microscopy provided an integrated picture concerning the dynamic properties and localization of EGF-receptor complexes in target cells. We anticipate that these and other new methods will be used to detect the interaction between EGF receptor and other membrane or cytoplasmic components which lead to the generation of the transmembrane signal mediated by EGF.

Finally, the generation of monoclonal antibodies against EGF receptor which induce both early and delayed effects of EGF[55] provides a powerful tool for future studies concerning the purification, localization and mode of action of EGF receptor in vitro and in situ.

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